Volume 258, number 1, 22–26 FEB 07823 November 1989

Animal and plant mitochondria contain specific thioredoxins

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Received 18 September 1989

Thioredoxins have been purified from pig heart and potato tuber mitochondria which differ in chromatographic behaviour, enzyme activating capacity, and slightly higher molecular mass ($M_r = 12\,500$) from the major thioredoxin(s) present in mitochondria-free fractions of the same tissue. Both mt-thioredoxins can serve as hydrogen donor for *E.coli* ribonucleotide reductase but only the plant protein activates spinach chloroplast NADP malate dehydrogenase in vitro. Mitochondrial target enzymes specifically activated by thioredoxin have not as yet been identified.

Cell organelle; Enzyme activation; Mitochondria; Thioredoxin; (Pig heart, Potato tuber)

1. INTRODUCTION

Thioredoxins are a group of small redox proteins which participate in many diverse biochemical processes, such as ribonucleotide reduction, protein disulfide reduction, light regulation of chloroplast enzymes, and sulfur metabolism [1–3]. Their universal distribution in bacteria and archaebacteria, animal and plant cells has been well documented. The proteins frequently occur in multiple species in the same organism. This is particularly evident in plants where up to 6 (or more) proteins, located inside and outside chloroplasts, can be found [4–6] but several thioredoxins, or thioredoxin genes were also observed in non-photosynthetic organisms like yeast [7] or *Corynebacterium nephridii* [8] and other bacteria. These isothioredoxins usually possess very similar molecular properties and a high degree of sequence homology. However, knowledge of the individual physiological specificities of various thioredoxins in a cell is scanty because most of them may be exchanged in heterologous in vitro assays without loss of activity [9].

One aspect of the multifunctional role of thioredoxins which is far from sufficiently known is their intracellular distribution. Thioredoxin has been detected by radioimmunoassay in all subcellular fractions of calf thymus and liver [10]. Immunohistochemical studies in rat tissues revealed an enrichment in the cytoplasm and at various membranes but gave confilicting results about the presence in nuclei and mitochondria [11,12]. In no case has a mammalian thioredoxin been purified from cell organelles. In plants, interest has focussed on the chloroplast thioredoxins and their light-dependent functions [4,5] whereas the nature of other leaf thioredoxins has remained obscure [13,14]. Only in a green alga, *Scenedesmus obliquus*, could we establish a specific cytoplasmic function of two extrachloroplastic plant thioredoxins, viz. the activation of algal ribonucleotide reductase [15].

In view of the endosymbiotic nature of cell organelles we reasoned that mitochondria should also possess their own thioredoxin(s). A protein with thioredoxin-typical properties could indeed be detected in heart mitochondria which differed from most other thioredoxins in its inability to stimulate the commonly used indicator enzyme, leaf NADP-malate dehydrogenase [16]. We here describe the purification and characteristics of this mammalian mitochondrial thioredoxin as well as a thioredoxin isolated from potato tuber mitochondria.

2. MATERIALS AND METHODS

Pig heart mitochondria were prepared and washed by differential centrifugation in 0.25 M sucrose solution containing 20 mM Tris-HCl buffer, pH 7.8, and 2 mM EDTA [17]. Potato tuber (*Solanum tuberosum*, cv. 'Sieglinde') mitochondria were obtained by density gradient centrifugation in 0.3 M sucrose solution containing 10 mM K-phosphate, pH 7.2, mM EDTA, 0.1% bovine serum albumin, and 28% Percoll (Pharmacia) [18].

Mitochondrial respiration and integrity were determined with an oxygen electrode [18]. Glucose-6-phosphate dehydrogenase and catalase activities were determined photometrically by standard procedures. Preparation and activity determination of ribonucleotide reductase from *E. coli* [19] (substrates: 0.14 mM [^{3}H]cytidine diphosphate and 2 mM dithiothreitol) and of NADP malate dehydrogenase from spinach leaves [20] were carried out as described before. For activation, the enzymes were incubated in the presence of thioredoxin for 30 or 60 min at 30°C. Electrophoresis on SDS-polyacrylamide (12%) gels was done in a Tris/Tricine (pH 8.3) buffer system [21].

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

mt-Thioredoxin from pig heart

Washed mitochondria obtained from 6 hearts (1500 g tissue) were suspended in 180 ml 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM dithiothreitol, and were disrupted by 3 × 30 s sonification. After centrifugation for 10 min at 39,000 × *g* the supernatant was rapidly heated to 80°C and then cooled in ice. Denatured proteins were removed by centrifugation and the yellow supernatant was concentrated to about 8 ml in Aquacide II. The solution was chromatographed on a column (2.8 × 53 cm) of Sephadex G-75 in the above described Tris buffer. Active fractions were further purified on a DEAE cellulose column (1 × 4.5 cm) which was eluted with a 0–0.2 M NaCl gradient in the same buffer.

mt-Thioredoxin from potato tuber

Mitochondria obtained from 10 kg potatoes were sonicated and centrifuged as above. The turbid supernatant was made 1% in streptomycin sulfate, the precipitate was removed after 1 h, and the supernatant subjected to heat denaturation as above. Gel filtration of the concentrated solution was carried out on a column (1 × 83 cm) of Sephadex G-100 in 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl. Active fractions were pooled and were applied to a column of DEAE cellulose (1 × 6.4 cm) and chromatographed in 20 mM Tris-HCl buffer, pH 8.6, with a 0–0.3 M NaCl gradient.

3. RESULTS

Because of the apparently ubiquitous distribution of thioredoxins the detection of an organelle-specific protein requires the isolation of organelles in high purity and high yield. Mitochondria were prepared from pig heart and potato homogenates by the optimum centrifugation techniques and washing cycles as described in section 2 [17,18]. The purified mitochondrial fractions were analyzed for respiratory activity and for the absence of cytoplasmic, plastid, and peroxisomal enzymes; by these criteria, both types of mitochondria used in our experiments were intact and contained less than 0.1% contaminating activities.

The mitochondria were lysed by sonication, subjected to heat (80°C) treatment, and the heat-stable extracts were analyzed for thioredoxin activity. In the absence of any known mitochondrial target system we selected a bacterial ribonucleotide reductase (from *E. coli*) as indicator enzyme which is the most unspecific and most reliable, albeit most difficult, thioredoxin assay in cases of limited crossreactivity [22]. The mitochondrial protein fractions stimulated CDP reduction 2–3-fold, which is a safe indication for the presence of thioredoxins. In fact, no other assay has been found for the determination of heart mt-thioredoxin (vide infra).

The heat-stable mitochondrial proteins were subjected to a typical thioredoxin purification protocol as summarized in table 1. Thioredoxin activity eluted from a Sephadex G-75 column in the 10–15 kDa molecular mass range. DEAE cellulose chromatography was effective in separating thioredoxin, which was bound, from basic cytochrome c, which passed through the anion exchange column; both proteins could not have been differentiated by gel filtration because of their almost identical molecular weights. As judged by SDS-polyacrylamide gel electrophoresis, the two mitochondrial thioredoxins were purified to apparent homogeneity by these procedures (fig.1), and possess a molecular mass around 12 kDa like most other thioredoxins.

It was essential to examine the organelle-specific nature of these proteins by comparison with the main (cytoplasmic) thioredoxin(s) present in the animal or plant tissues. In experiments not detailed here, the supernatants remaining after isolation of the heart or potato mitochondria were heat-treated and the heat-stable proteins were subjected to further purification in close analogy to the mitochondrial fractions. In these preparations it was possible to use the spectrophotometric NADP-MDH activation assay for routine thioredoxin determinations. From pig heart we obtained one other thioredoxin (to be described elsewhere) which in its properties corresponds to the proteins previously characterized in bovine, rat, and rabbit tissues [23–25] and is thought to be the regular,

Table 1

Purification of thioredoxins from mitochondria

Purification step	Total protein (mg)	Total activity (nmol dC · h $^{-1}$)	Specific activity (nmol dC · h $^{-1}$ · mg $^{-1}$)
Pig heart[a]			
supernatant after heat denaturation	29.2	284	9.7
Sephadex G-75 active fractions	5.80	102	17.6
DEAE cellulose active fractions	1.31	197	150
Potato tuber[b]		(nmol NADP · min $^{-1}$)	(nmol NADP · min $^{-1}$ · mg $^{-1}$)
supernatant after heat denaturation	26.1	117	4.8
Sephadex G-100 active fractions	5.1	92	18.1
DEAE cellulose active fractions	0.58		145

[a]Preparation starting from 342 mg of soluble mitochondrial protein; thioredoxin activity determination is not possible in the crude lysate

[b]Preparation starting from 134 mg of soluble mitochondrial protein; activity determination with chloroplast NADP-MDH

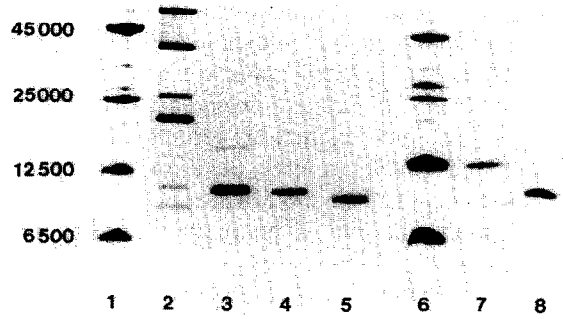

Fig.1. SDS-polyacrylamide gel electrophoresis of heart and potato thioredoxins. (Lanes 1 and 6) Marker proteins (from top: ovalbumin, chymotrypsinogen, cytochrome c, aprotinin). (Lane 2) Heat-stable proteins from pig heart mitochondria; (lane 3) eluate from Sephadex G-75; (lane 4) heart mt-thioredoxin after DEAE cellulose chromatography; (lane 5) heart cytoplasmic thioredoxin, purified separately from mitochondria-free heart homogenate. (Lane 7) Purified mt-thioredoxin from potato mitochondria; (lane 8) potato thioredoxin I, purified separately from mitochondria-free tissue homogenate.

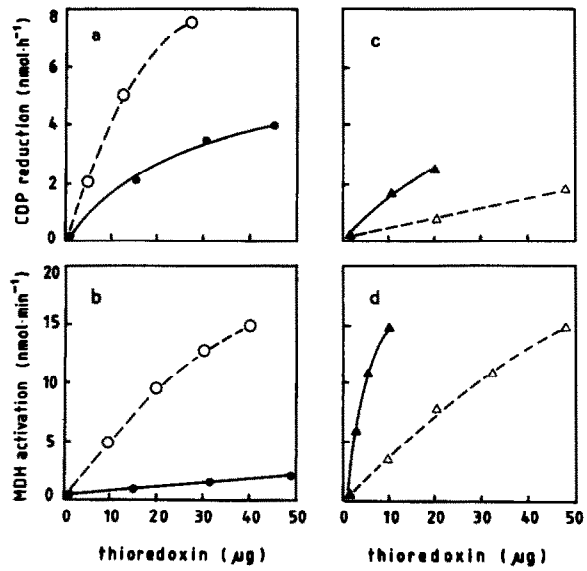

Fig.2. Enzyme-activating activities of the thioredoxins isolated from pig heart (a,b) and potato tuber tissue (c,d). Thioredoxins were purified to homogeneity and reacted with *E. coli* ribonucleotide reductase (upper panel, a and c) and leaf NADP-malate dehydrogenase (lower panel, b and d). Filled symbols = mt-thioredoxins; open symbols = non-mitochondrial cellular thioredoxins.

cytoplasmic thioredoxin. DEAE cellulose chromatography of the mitochondria-free potato tuber homogenate resolved three protein fractions with NADP-MDH stimulating activity and molecular masses around 12 kDa, which together closely resemble the thioredoxin pattern found in other non-photosynthetic plant tissues such as soy bean and wheat seeds [6,26]; however, the cytoplasmic or (pro)plastid origin of these thioredoxins was not analyzed further.

Table 2 summarizes properties which differentiate the mitochondrial and the other cellular thioredoxins. Both mt-thioredoxins exhibit slightly higher molecular weights on denaturing electrophoresis gels (fig.1), and they require higher ionic strength during gradient elution from otherwise identical anion exchange columns; these differences were reproduced in cochromatography experiments in which separately purified proteins had been recombined (not shown). Other physical properties such as heat stability and the increase in fluorescence emission at 345 nm following reduction with dithiothreitol were the same for all the new thioredoxins described here.

The purified mt-thioredoxins are also clearly distinguished from their cellular counterparts by specific enzyme-stimulating activities (fig.2). Thus, the mammalian mt-thioredoxin is less active in the ribonucleotide reduction assay, and its stimulatory activity towards chloroplast NADP-MDH is barely detectable. In contrast, the protein isolated from potato mitochondria is a rather active thioredoxin which exceeds the other plant thioredoxins in activity in both enzyme systems. These differences between animal and plant thioredoxins are not per se surprising as long as physiological, mitochondrial target enzymes have not been identified.

4. DISCUSSION

We have demonstrated the existence of 12–12.5 kDa proteins in mammalian and plant mitochondria which

Table 2

Properties of mitochondrial and non-mitochondrial thioredoxins from pig heart and potato tuber

Source of thioredoxin	Heart		Potato	
	mt	non-mt	mt	non-mt[b]
molecular mass	12 200	12 000	12 500	12 000
elution from DEAE-cellulose (mM NaCl)	120	60	70	<20
MDH activation[a] (nmol NADP · $min^{-1} \cdot \mu g^{-1}$)	0.072	0.48	1.7	0.3
ribonucleotide reduction[a] (nmol deoxycytidine · $h^{-1} \cdot \mu g^{-1}$)	0.118	0.402	0.178	0.024

[a] Specific thioredoxin activities were calculated in the linear range of enzyme activation with subtraction of the unstimulated (no thioredoxin) enzyme activity

[b] The data refer to homogeneous thioredoxin I, i.e. the predominant and most active species out of 3

exhibit the general properties common to all thioredoxins but can be distinguished from the major thioredoxins present in heart and potato homogenates. Because the mitochondria used in these preparations were highly purified, intact organelles we identify these proteins as mt-thioredoxins. We have tested the new proteins as hydrogen donor in glutathione-dependent ribonucleotide reductase assays [25] but found no glutaredoxin activity.

The mt-thioredoxins may previously have been overlooked in preparative and cytochemical thioredoxin studies because of their low amount in total tissue homogenates, limited immunological crossreactivity, and the lack of specific enzyme assays. It is possible that lesser fractions of thioredoxin activity observed in addition to highly active, regular thioredoxins in both plant and mammalian cell extracts [15,26–28] represent thioredoxins of mitochondrial origin; the nature of such minor thioredoxin fractions has not been clarified anywhere.

The identification of one more group of eukaryotic thioredoxins of apparently general occurrence seriously complicates previous assignments of thioredoxin fractions found in plant tissue extracts to the f-, m-, or h-categories [2,4,14]. These functional specifications are based primarily on the in vitro activation of chloroplast enzymes whereas the intracellular localization of the various thioredoxins was frequently not determined, and typical cytoplasmic reactions like ribonucleotide reduction were not even considered.

The physiological role of mt-thioredoxins is unknown. Dithiols have been implicated in several mitochondrial processes including oxidative phosphorylation [29] but participation of thioredoxins has so far found little attention. Activation of a urea cycle enzyme, carbamoyl phosphate synthetase, in rat liver is the only reported case [30]. Thioredoxin activation of ATP synthesis is a distinct possibility, and ought to be tested, in view of the thiol modulation and interaction of thioredoxin with the proton ATPase (CF_0-CF_1) of chloroplasts [31] and the general similarities of energy-producing mechanisms in chloroplasts and mitochondria. On the other hand, mammalian and plant mitochondria differ substantially from each other, with the plant organelles being closer to the eubacterial line of descent [32]. Extra functions of mt-thioredoxins may therefore be envisaged in plant cells; for example, in photorespiration which requires cooperation between chloroplasts, containing the light-actuated thioredoxin/enzyme systems, and mitochondria, which have the dithiol-dependent glycine oxidation reaction [33,34]. It may be pertinent in this context that the plant mt-thioredoxin is more active than its mammalian counterpart and is able to activate chloroplast NADP-MDH in vitro. Moreover, a preliminary characterization of mitochondrial and chlorolast thioredoxins in soybean leaves (I. Häberlein and A. Buch, unpublished) indicates that the activities of these two organelle-specific proteins are of the same order of magnitude. We are currently exploring mitochondrial enzymes for potential thioredoxin effects.

Acknowledgements: This study has been supported by Deutsche Forschungsgemeinschaft, SFB 305 (Ökophysiologie), and by Fonds der Chemischen Industrie. We thank Professor H.W. Heldt, Göttingen, and Professor A. Holmgren, Stockholm, for stimulating discussions.

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